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Rinderpest Virus Phosphoprotein Gene Is a Major Determinant of Species-Specific Pathogenicity

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We previously demonstrated that the rinderpest virus (RPV) hemagglutinin (H) protein plays an important role in determining host range but that other viral proteins are clearly required for full RPV pathogenicity to be manifest in different species. To examine the effects of the RPV nucleocapsid (N) protein and phosphoprotein (P) genes on RPV cross-species pathogenicity, we constructed two new recombinant viruses in which the H and P or the H, N, and P genes of the cattle-derived RPV RBOK vaccine were replaced with those from the rabbit-adapted RPV-Lv strain, which is highly pathogenic in rabbits. The viruses rescued were designated recombinant RPV-lapPH (rRPV-lapPH) and rRPV-lapNPH, respectively. Rabbits inoculated with RPV-Lv become feverish and show leukopenia and a decrease in body weight gain, while clinical signs of infection are never observed in rabbits inoculated with RPV-RBOK or with rRPV-lapH. However, rabbits inoculated with either rRPV-lapPH or rRPV-lapNPH became pyrexic and showed leukopenia. Further, histopathological lesions and high virus titers were clearly observed in the lymphoid tissues from animals infected with rRPV-lapPH or rRPV-lapNPH, although they were not observed in rabbits infected with RPV-RBOK or rRPV-lapH. The clinical, virological, and histopathological signs in rabbits infected with the two new recombinant viruses did not differ significantly; therefore, the RPV P gene was considered to be a key determinant of cross-species pathogenicity.

Rinderpest virus (RPV) is a single-stranded, negative-sense RNA virus classified in the genus *Morbillivirus* in the family *Paramyxoviridae,* order *Mononegavirales*. RPV is antigenically closely related to other members of this genus, which include human measles virus (MV), peste des petits ruminants virus in sheep and goats, canine distemper virus (CDV), and phocid distemper virus, which cause diseases in carnivore species, including seals. Since the late 1980s, novel CDV and related morbillivirus infections have occurred worldwide and have caused fatal disease in several species previously considered immune to these viruses, including hyenas and various marine mammals (5, 6). Two recent CDV epidemics in large cats have been reported, one in California and the other in the Serengeti, Tanzania (1, 25a). Both were characterized by significant fatality rates (23 and 30%, respectively). Recently, two new emerging paramyxoviruses that were identified in cases of severe respiratory and encephalitic diseases in animals and humans have been described; they are now known as Hendra virus (HeV) and Nipah virus (NiV) (12). Hendra virus emerged in 1994 and was transmitted to humans by close contact with horses; Nipah virus emerged in 1999 and was passed from pigs to humans. Both are unusual among the paramyxoviruses in their abilities to infect and cause potentially fatal disease in a number of host species, including humans, although their natural host is the fruit bat. From these incidents it is clear that a high probability of cross-species infection by paramyxoviruses exists, and so the determinants of cross-species pathogenicity following infection with these viruses should be a high priority for research. As a first step toward understanding these mechanisms, we studied the role of the hemagglutinin (H) protein gene of RPV as a pathogenic determinant in a rabbit model, since virus entry was considered one of the key elements determining cross-species infectivity (29). From these experiments it was clear that the H protein of the lapinized strain played an important role in allowing infection to occur but was not a factor determining pathogenicity for rabbits. It was then considered likely that one of the internal virus protein genes, or a combination of these genes from the lapinized virus, was essential to produce clinical disease in rabbits. The results of experiments in which the phosphoprotein (P) gene (recombinant RPV-lapPH [rRPV-lapPH]), alone or together with the nucleocapsid (N) protein gene (lapNPH), from RPV-Lv was additionally swapped into the RBOK backbone are presented in this report.

MATERIALS AND METHODS

Cells. B95a cells, which are highly susceptible to RPV infection (18), were propagated in RPMI 1640 medium (Sigma, St. Louis, Mo.) supplemented with 5% fetal calf serum in a humidified atmosphere containing 5% CO_2. RPMI 1640 supplemented with 2% fetal calf serum was used as maintenance medium. The antibiotics benzylpenicillin (100 U/ml) and streptomycin (100 U/ml) were used in all media.

Construction of the full-length cDNA of RPV. The RPV-Lv strain, which is a highly virulent for rabbits and was obtained by virus cloning from the RPV-L strain (M. Shiotani et al., submitted for publication), was used in this study, along with the rRPV-RBOK virus rescued from a full-length DNA copy (cDNA) of the genome of the RBOK vaccine strain (3). Virus was also rescued from a plasmid carrying the full genome of the rRPV-RBOK virus with the H protein gene derived from RPV-Lv (rRPV-lapH) (29). This plasmid was used for constructions of full-length cDNAs in which the P gene alone or the N and P genes

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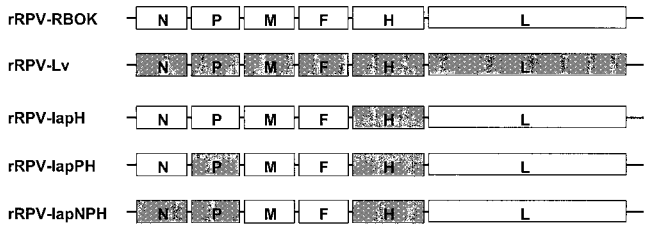

FIG. 1. Schematic representation showing constructions of recombinant viruses. White and dark bars represent the genes of RPV-RBOK strain and RPV-Lv, respectively.

combined were derived from RPV-Lv. To enable replacement of the P gene in the RPV-lapH cDNA, an FseI site was introduced just before the N-P intergenic region and a PmeI site was introduced just before the P-M intergenic sequence. The P gene from RPV-Lv was amplified by reverse transcription-PCR from total RNA of RPV-Lv-infected B95a cells by using oligonucleotide primers with these restriction enzyme sites and was inserted in place of the normal P gene in the RPV-lapH cDNA to rescue a virus designated rRPV-lapHP. Similarly, the N gene was inserted into the latter virus by using a ClaI site at the beginning of the N gene of strain RPV-RBOK and the FseI site at the N-P intergenic region.

Rabbits, virus inocula, and samples. Two-month-old female albino rabbits (JW-NIBS strain) with an average body weight of 1.6 kg, which are highly sensitive to RPV-L (22), were obtained from the Nippon Institute for Biological Science (Tokyo, Japan). One milliliter each of the rRPV-RBOK, rRPV-lapH, rRPV-lapPH, rRPV-lapNPH, and RPV-Lv viruses, diluted to 10^4 50% tissue culture infective doses ($TCID_{50}$)/ml with maintenance medium, was inoculated into rabbits, using two rabbits per experiment. The experiment was repeated twice. One control rabbit was inoculated with 1 ml of maintenance medium. The rabbits were euthanatized at 4 days postinoculation (dpi) by using Dormitor (Orion, Espoo, Finland) and Dormicum (Roche, Basel, Switzerland), and selected tissues were collected for further investigation.

Clinical investigations. Inoculated rabbits were examined daily, and rectal temperatures and body weights were recorded. Total white blood cell (WBC) counts in the peripheral blood of the rabbits were determined with a commercial kit (Unopette Test 58.56; Becton Dickinson).

Virological investigations. The rabbits were euthanatized at 4 dpi, and the lymphoid tissues, consisting of the spleen, Peyer's patches, mesenteric lymph nodes, and appendices, were collected and weighed. Virus infectivity titers in 10% (wt/vol) homogenates of these tissues were determined in B95a cells and expressed as $TCID_{50}$ per milliliter.

Histopathological examination. The various lymphoid tissues and other organs removed at autopsy were fixed in 10% formalin, dehydrated, and embedded in paraffin by using routine techniques. Thin sections were stained with hematoxylin and eosin.

RESULTS

Rescue of rRPV-lapPH and rRPV-lapNPH. rRPV-lapPH and rRPV-lapNPH were successfully rescued from their respective full-length cDNAs in 293 cells (Fig. 1). After 3 days of coculture of the transfected 293 cells with B95a cells, a number of small syncytia were observed. No difference in virus phenotype was observed in B95a cells infected with these new recombinant viruses, and the cytopathic effect was similar to that shown by rRPV-lapH and rRPV-RBOK (data not shown).

Clinical signs following virus inoculation into rabbits. Rabbits inoculated with the RPV-Lv strain show severe disease, with clinical signs such as pyrexia, leukopenia, and failure to gain body weight. Eight rabbits (two per virus strain) were inoculated with the four strains of RPV, and the experiment was carried out three times. The clinical disease seen with each virus type was very reproducible, as shown in Fig. 2. Rabbits inoculated with $10^4 TCID_{50}$ of RPV-Lv per ml became pyrexic from 1 dpi, while body weight loss and leukopenia were observed from 2 dpi. Rabbits inoculated with either rRPV-RBOK or rRPV-lapH showed no observable clinical signs of infection, as observed in our previous study (29). In contrast, rabbits inoculated with rRPV-lapPH or rRPV-lapNPH showed high fever on 2 dpi and also mild leukopenia, although body weight loss was not observed.

Virus growth in lymphoid tissues. The virus present in each of the lymphoid tissues examined was determined by titration in B95a cells, and the data are summarized in Table 1. High virus titers, ranging from 3.0 to 4.9 log $TCID_{50}$/ml, were detected in the mesenteric lymph nodes, superficial lymph nodes, appendices, spleens, and Peyer's patches of all rabbits inoculated with RPV-Lv. In contrast, no virus was detected in lymphoid tissues of rabbits inoculated with either rRPV-RBOK or rRPV-lapH, as previously noted (29). High virus titers were detected in all of the tissues of one rabbit inoculated with rRPV-lapPH. In the other rabbit, virus was detected only in the spleen and in Peyer's patches. Similarly, high virus titers were measured in the lymphoid tissues collected from rabbits infected with rRPV-lapNPH.

Histopathology of lymphoid tissues. All of the lymphoid tissues collected were examined for gross pathological changes, and those from rabbits inoculated with rRPV-RBOK were normal in every respect. In agreement with the clinical disease seen, severe necrotic lesions, infiltration of inflammatory cells, and multinuclear giant cells, which are characteristics of RPV-Lv infection, were also observed in tissues from rabbits infected with rRPV-lapPH or rRPV-lapNPH (Fig. 3). The "starry-sky" effect, which was observed in the spleens of rabbits infected with rRPV-lapH (29), was also seen in the present experiments.

DISCUSSION

The H protein of a neuroadapted mouse strain of MV has been shown to determine virus neurotropism (11), and in a previous study we showed that the H protein of RPV-Lv was required to allow the bovine cell culture-derived PRV-RBOK vaccine strain to infect rabbits; however, the virus produced was not capable of causing disease in that species (29). It was

TABLE 1. Virus titers in rabbit lymphoid tissues

Inoculated virus	Rabbit no.	Virus titer (log_{10} $TCID_{50}$/ml) at 4 dpi in:				
		Mesenteric lymph node	Superficial lymph node	Appendix	Spleen	Peyer's patch
RPV-Lv	1	4.8	3.9	4.9	3.0	3.5
	2	4.7	3.1	4.0	3.0	3.9
RPV-RBOK	3	—[a]	—	—	—	—
RPV-lapH	4	—	—	—	—	—
	5	—	—	—	—	—
RPV-lapHP	6	—	—	—	1.5	2.5
	7	3.8	2.0	4.0	3.0	4.0
RPV-lapNHP	8	2.1	—	3.8	1.0	4.0
	9	2.0	—	1.8	2.0	2.7

[a] —, not detected.

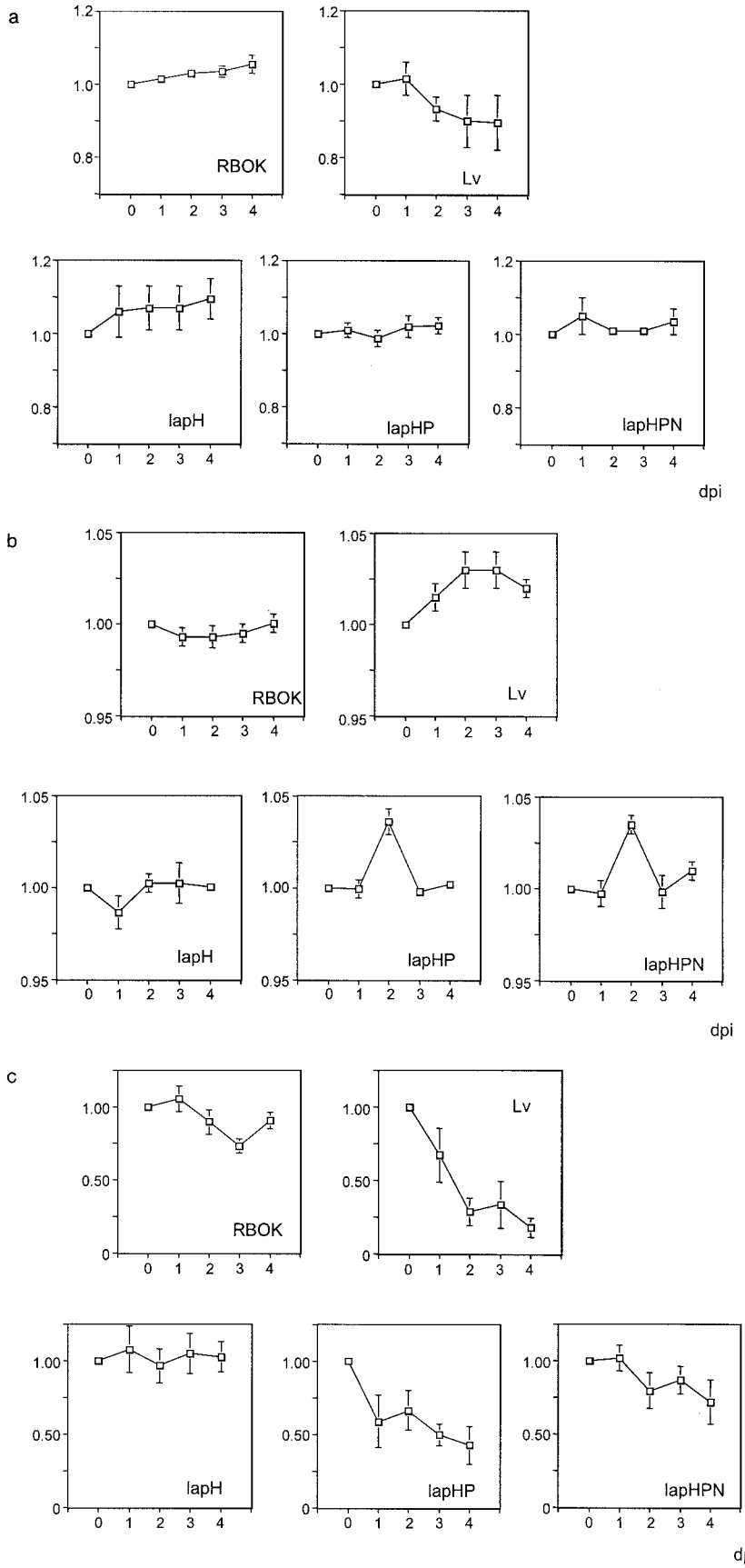

FIG. 2. Clinical signs following virus inoculation into rabbits. All viruses were inoculated intravenously into rabbits in a 1.0-ml volume containing $10^{4.0}$ $TCID_{50}$ of the respective virus. Body weight (a), rectal temperature (b), and WBC count (c) were recorded daily throughout the experiment. The levels have been standardized, with those on the first day being 1. Values are means ± standard deviations from six rabbits.

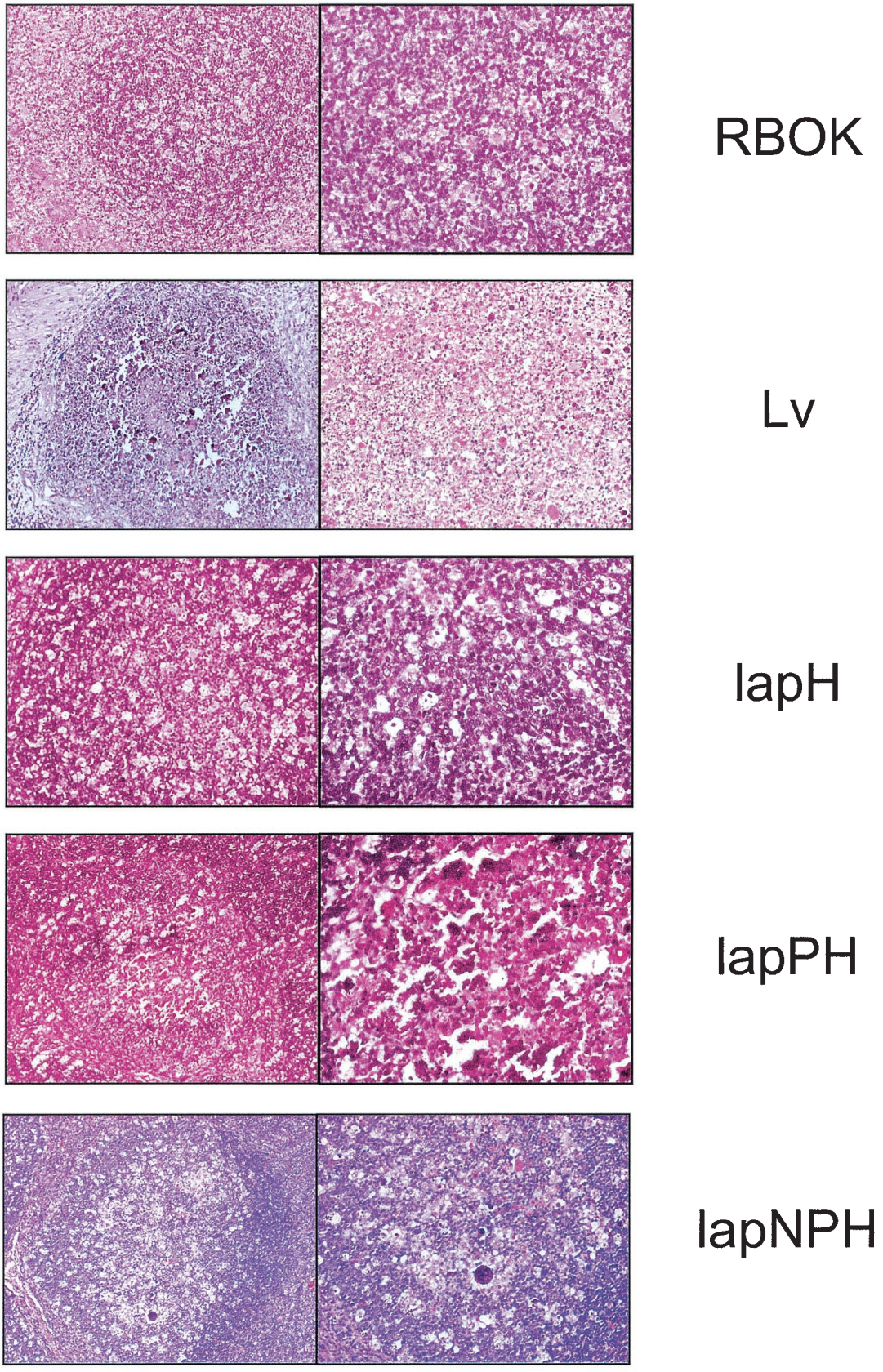

FIG. 3. Histopathology in lymphoid tissues. Thin sections of lymphoid tissues were stained with hematoxylin and eosin. Representative lesions observed in Peyer's patches are shown for each virus. Magnifications, ×200 (left panels) and ×400 (right panels).

therefore clear that virus entry alone was not sufficient to ensure that a virus is pathogenic in the nonnatural host. We then focused on two of the internal virus protein genes, the N and P genes, as possible determinants of virus virulence for rabbits, as the other envelope-associated proteins, the F and M proteins were not likely to be involved in this function. The genomes of single-stranded, negative-sense RNA viruses are tightly encapsidated by the N protein, and the N-RNA complex serves as the template for both virus transcription and replication. Within the virion, the RNA-dependent RNA polymerase (L protein) is associated with the N-RNA template through an interaction with the P protein to form the transcribing ribonucleoprotein (RNP) complex (21). Upon entry, virus transcription and replication of the RNP take place in the cytoplasm of infected cells. During transcription (as opposed to replication, where a full-length antigenome RNA is transcribed), the gene start and stop signals are recognized, and monocistronic, capped, and polyadenylated mRNAs for each virus protein are produced. The intracellular concentration of unassembled N protein, which exists as a soluble N-P complex, is considered one factor which regulates the switch from transcription to replication (2, 7, 19, 24).

The P proteins of paramyxoviruses are known to perform multiple functions during virus replication, and many of these functions are mediated through its association with the N and L proteins. The P proteins of negative-stranded RNA viruses are activated by phosphorylation and, in association with the N and L proteins and genome RNA, form the replicase complex, which is essential for progeny virus production. It has been shown for vesicular stomatitis virus and Sendai virus that the N protein must be complexed with P to keep it soluble for RNA encapsidation and prevent the N protein from aggregating to form RNP-like structures lacking RNA (8, 9, 20). In addition, Sendai virus P protein was shown to form tetramer (27).

We succeeded in rescuing recombinants in which the H and P genes or the N, H, and P genes of the RPV-RBOK strain were replaced with those derived from the RPV-Lv strain (rRPV-lapPH and rRPV-lapNPH), and we compared their pathogenicities for rabbits with those of the two parent strains and the rRPV-LapH recombinant, which was previously rescued. While neither rRPV-RBOK nor rRPV-lapH was virulent in rabbits, those inoculated with either RPV-Lv, rRPV-lapPH, or rRPV-lapNPH became pyrexic and showed a significant reduction in WBC counts. While rRPV-lapH could not be detected in homogenates of lymphoid organs from infected rabbits, high titers of both rRPV-lapPH and rRPV-lapNPH were detected in almost all of the lymphoid tissues from infected rabbits analyzed. These data supported a role for the P gene as an important factor determining efficient species-specific virus replication. Only the starry-sky effect, which indicated the occurrence of immune responses such as activation of macrophages and propagation of lymphocytes (13), was observed in lymphoid tissues of rRPV-lapH-infected rabbits, which, as reported previously, showed not other observable signs of infection.

Since the replication of rRPV-lapPH and rRPV-lapNPH occurred to similar degrees in rabbit organs, species-specific interaction of the RPV-Lv N and P proteins does not appear to be required for pathogenesis, and other, possibly host cell, factors may be implicated. An important fact to bear in mind is that the P genes of most paramyxoviruses, including all morbilliviruses, additionally code for the two nonstructural proteins C and V (19), which are not essential for replication in tissue culture cells (4, 25, 26) but which are known to be virulence determinants through their ability to counteract the innate immune responses to virus infection. Compared to the parent MV, the V-defective virus causes milder clinical symptoms and lower mortality rates in a human CD46-transgenic mouse model (23) and prolonged thymocyte survival in a mouse-engrafted human thymus-liver implant model (28). In particular, C and V have been shown to be necessary to counteract the effects of interferon induction in response to virus infection (10, 14–17). The identities between predicted amino acid sequences of the P, C, and V proteins of RPV-RBOK and those of RPV-L were 80 to 86% (unpublished data). Which protein among these three plays the key role in cross-species pathogenicity is now under investigation.

While the replacement of the P gene of RPV-RBOK, along with the H gene, with those from RPV-Lv resulted in a level of virulence for rabbits that was very close to that caused by RPV-Lv itself, it was not identical in that there was no decrease in the body weights of infected rabbits, and so another virus gene(s), most probably the L gene, might also be required to confer full pathogenicity in this species. Experiments to produce an rRPV-lapNPHL recombinant to test this hypothesis and to investigate the host cell factors that may interact with P, and the P gene-associated C and V proteins, to determine virulence for rabbits are under way.

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